PATENT CLAIMS

1. A method for constructing a RP-II protease variant, wherein the variant has at least one altered property as compared to a parent RP-II protease, which method comprises:

a) analyzing the three-dimensional structure of the RP-II protease to identify, on the basis of an evaluation of structural considerations, at least one amino acid residue or at least one structural region of the RP-II protease, which is of relevance for altering said property;

b) modifying the DNA of the polynucleotide encoding the parent to construct a polynucleotide encoding a variant RP-II protease, which in comparison to the parent RP-II protease, has been modified by deletion, substitution or insertion of the amino acid residue or structural part identified in i) so as to alter said property;

c) expressing the variant RP-II protease in a suitable host, and

d) testing the resulting RP-II protease variant for said property.

2. A method of producing a BLC like RP-II protease variant, wherein the variant has at least one altered property as compared to a parent BLC like RP-II protease, which method comprises:

a) producing a model structure of the parent BLC like RP-II protease on the three-dimensional structure of BLC,

b) comparing the model three-dimensional structure of the parent BLC like RP-II protease to the BLC structure by superimposing the structures through matching the CA, CB, C, O, and N atoms of the active site residues,

c) identifying on the basis of the comparison in step a) at least one structural part of the parent BLC like RP-II protease, wherein an alteration in said structural part is predicted to result in an altered property;

d) modifying the nucleic acid sequence encoding the parent BLC like RP-II protease to produce a nucleic acid sequence encoding at least one deletion or substitution of one or more amino acids at a position corresponding to said structural part, or at least one insertion of one or more amino acid residues in positions corresponding to said structural part;

e) performing steps c) and d) iteratively N times, where N is an integer with the

value of one or more;

f) preparing the variant resulting from steps a) - e);

g) testing the stability of said variant; and

h) optionally repeating steps a) - g) recursively; and

i) selecting a RP-II protease variant having at least one altered property as compared to the parent RP-II protease.

j) expressing the modified nucleic acid sequence in a host cell to produce the variant RP-II protease;

k) isolating the produced protease;

l) purifying the isolated protease and

m) recovering the purified RP-II protease variant.

3. The method of claim 2, wherein step (c) identifies amino acid residue positions located at a distance of 10Å or less to the ion-binding site of the RP-II protease parent, preferably positions located at a distance of 6 Å or less.

4. The method of claim 2, wherein step (c) identifies amino acid residue positions in the RP-II protease parent, the modification of which provides for the removal of the ion binding site by modification of at least one of the positions identified.

5. The method of claim 2, wherein step (c) identifies amino acid residue positions in highly mobile regions of the RP-II protease parent.

7. The method of claim 2, wherein step (c) identifies amino acid residue positions in mobile regions of the RP-II protease parent.

8. The method of claim 2, wherein step (c) identifies amino acid residue positions in the parent RP-II protease, the modification of which may create at least one disulfide bridge by insertion of or substitution with at lease one Cys residue.

9. The method of claim 2, wherein steps (c) and (d) provide for constructing a variant of a parent RP-II protease having a modified surface charge distribution by:

c') identifying, on the surface of the parent RP-II protease, at least one charged amino acid residue;

d') modifying the charged residue identified in step (a) through deletion or substitu-

tion with an uncharged amino acid residue;

10. The method of claim 2, wherein steps (c) and (d) provide for constructing a variant of a parent RP-II protease having a modified surface charge distribution by:

c") identifying, on the surface of the parent RP-II protease, at least one position being occupied by an uncharged amino acid residue;

d") modifying the charge in that position by substituting the uncharged amino acid residue with a charged amino acid residue or by insertion of a charged amino acid residue at the position.

11. The method of claim 2, wherein steps (c) and (d) provide for constructing a variant of a parent RP-II protease having a modified surface charge distribution by:

c'") identifying, on the surface of the parent RP-II protease, at least one charged amino acid residue;

d'") substituting the charged amino acid residue identified in step (a) with an amino acid residue having an opposite charge.

12. The method of claim 2, wherein step (c) identifies amino acid residue positions in the parent RP-II protease, the modification of which to Pro may create a RP-II protease variant exhibiting improved stability.

13. The method of claim 2, wherein step (c) identifies amino acid residue positions in the parent RP-II protease at a distance of less than 10Å from the active site residues.

14. The method of one or more of claims 2 to 13, wherein N in step (e) is an integer between 1 and 50, 45, 40, 35, 30, 25, 20, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2.

15. A RP-II protease variant comprising at least one modification in an amino acid residue in a position located at a distance of 10Å or less to the ion-binding site, preferably positions located at a distance of 6 Å or less.

16. The variant of claim 15, wherein modifications are made in at least one of the positions: 1, 2, 3, 4, 5, 6, 7, 8, 143, 144, 145, 146, 158, 159, 160, 161, 162, 194, 199, 200, and 201, preferably positions 2, 3, 4, 5, 6, 7, 144, 159, 160, and 161, and especially the modifications D7E and D7Q in BLC (SEQ ID NO: 2), where the positions refer

to BLC or corresponding positions.

17. The variant of claims 15 or 16, wherein the modification comprises the substitution of a positively charged amino acid residue with a neutral or negatively charged residue, or the substitution of a neutral residue with a negatively charged residue or the deletion of a positively charged or neutral residue.

18. The variant of claim 15, wherein the ion binding site is removed by modification in at least one of the positions corresponding to positions 144 and or 161 of BLC, especially the modifications H144R and/or D161R,K+H144Q,N in BLC (SEQ ID NO:2).

19. A RP-II protease variant comprising at least one modification in an amino acid residue in highly mobile regions in at least one of the positions corresponding to positions 26-31 (26, 27, 28, 29, 30, and 31); 89-91 (89, 90, and 91); 216-221 (216, 217, 218, 219, 220, and 221) of BLC.

20. The variant of claim 19, wherein the parent is BLC and the modification comprises G30A and/or G91A.

21. A RP-II protease variant comprising at least one modification made in mobile regions in at least one of the positions corresponding to positions 51-56, (51, 52, 53, 54, 55, 56), 88-94, (88, 89, 90, 91, 92, 93, 94), 118-122 (118, 119, 120, 121, 122), and 173-183 (173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183) of BLC, preferably the regions 51-56 and 118-122.

22. A RP-II protease variant having at least one disulfide bridge provided by modifying the amino acid residues in positions 128 and 145 in BLC or corresponding positions to Cys, preferably the substitutions S145C and T128C in BLC or corresponding positions.

23. A RP-II protease variant having a modified surface charge distribution in comparison to the parent RP-II protease comprising modifications in at least one of the positions corresponding to positions 7, 17, 95, 109, 143, 174, 209, 216, of BLC, especially the modifications

D7N, S, T
Y17R, K, H
Y95R, K, H
T109R, K, H
Q143R, K, H
Q174R, K, H
E209Q, N
N216R, K, H
in BLC (SEQ ID NO. 1)

24. A RP-II protease variant exhibiting improved stability in comparison to the parent RP-II protease comprising at substitution to Pro in at least one of the positions corresponding to positions 18, 115, 185, 269 and 293 in BLC, especially one or more of the substitutions: T60P, S221P, G193P, V194P in BLC (SEQ ID NO. 1).

25. A RP-II protease variant comprising modifications in amino acid residues in positions corresponding to positions 1, 8, 22-35 (22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35), 42-58 (42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58), 82-100 (82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100), 129-135 (129, 130, 131,132, 133, 134, 135), 141-142, 153-156 (153, 154, 155, 156), 158, 161-171 (161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171), 188-193 (188, 189, 190, 191, 192, 193), 195,, 201-207 (201, 202, 203, 204, 205, 206, 207), 210, 213-214, 217 in BLC at a distance of less than 10Å from the active site residues.

26. The RP-II protease variant of any of the claims 15 to 25, further comprising at least one of the modifications (i) amino acid residues in positions that form part of an Asn-Gly sequence being modified by deletion or substitution, preferably with Asp, Gln, Ser, Pro, Thr, or Tyr; (ii) amino acid residues in positions that occupied by a Trp being modified by substitution with Phe, Thr, Gln or Gly; (iii) amino acid residues in positions that are occupied by Glu or Asp being modified by substitution with Ala; (iv) amino acid residues in positions that are in positions that are the 1st or 2nd position following a position occupied by a Glu or Asp residue being modified by substitution with a Pro; or (v) amino acid residues in positions that are occupied by a Met being modified by deletion or substitution, preferably with Ser or Ala.

27. The RP-II protease of any of claims 15 to 26 that is modified in a number of positions ranging from at least one and up to 50 positions, or from 1 to 45 positions, or from 1 to 40 positions, or from 1 to 35 positions, or from 1 to 30 positions, or from 1 to 25 positions, or from 1 to 20 positions, or from 1 to 15 positions, or from 1 to 14 positions, or from 1 to 13 positions, or from 1 to 12 positions, or from 1 to 11 positions, or from 1 to 10 positions, or from 1 to 9 positions, or from 1 to 8 positions, or from 1 to 7 positions, or from 1 to 6 positions, or from 1 to 5 positions, or from 1 to 4 positions, or from 1 to 3 positions, or from 1 to 2 positions, such modifications comprising substitutions, deletions, insertions and combinations thereof in the indicated number of positions.

28. An isolated polynucleotide comprising a nucleic acid sequence, which encodes for a RP-II protease variant defined or produced in any of the preceding claims.

29. The polynucleotide of claim 28, wherein the nucleic acid sequence has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% homology with the nucleic acid sequence shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, or SEQ ID NO:15.

30. An isolated nucleic acid construct comprising a nucleic acid sequence as defined in any of claims 28-29, operably linked to one or more control sequences capable of directing the expression of the polypeptide in a suitable expression host.

31. A recombinant host cell comprising the nucleic acid construct of claim 30.

32. A method for producing the RP-II variant defined or produced in any of claims 1 to 27 the method comprising:

a) cultivating the recombinant host cell of claim 31 under conditions conducive to the production of the RP-II protease variant; and

b) recovering the variant.

33. A detergent composition comprising a RP-II protease variant defined or produced in any of claims 1 to 27.

34. Use of a RP-II protease variant defined or produced in any of claims 1 to 27 for

washing or cleaning purposes.

35. Use of a RP-II protease variant defined or produced in any of claims 1 to 27 for processing food.

36. Use of a RP-II protease variant defined or produced in any of claims 1 to 27 for processing feed.

37. Use of a RP-II protease variant defined or produced in any of claims 1 to 27 for the treatment of hides.